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Intracytoplasmic morphologically selected sperm injection

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INTRODUCTION

Since its introduction, intracytoplasmic sperm injection (ICSI) has revolutionized the approach to male infertility by offering treatment to couples who were previously excluded from conventional in vitro fertilization (IVF).

Poor-quality samples with severe impairment of sperm count, motility, and morphology turned to be unexpectedly suitable to achieve pregnancy and deliver healthy babies (1). According to some authors, the only requirement of that approach is the presence of at least one motile spermatozoon, while its morphology does not seem to have any correlation with ICSI outcomes (2–5) given that fertilization can occur even in cases of total teratozoospermia (6), globozoospermia (7,8), and megalozoospermia (9). On the other hand, fertilization, pregnancy, implantation (9,10), embryo quality (11–13), and blastocyst formation (11,14) rates are negatively affected by severe morphological anomalies. Even if there is a tendency among embryologists to select for injection into the ooplasm only the most good-looking sperm and discard the most distorted ones (i.e., round, large, or tapered),visual assessment under 200–400× magnification can actually identify mostly rough alterations in sperm shape and size (15). However, it overlooks a variety of head defects (16–18), which could be indicative of impaired sperm function and DNA integrity, as is significantly frequent in cases of oligo-astheno-teratozoospermia (OAT) (19–25). Moreover, ICSI involves the use of a spermatozoon that would never be able to penetrate the zona pellucida because of structural defects whose severity seems to be related to the incidence of chromosome aneuploidies (26). Hence, its introduction into clinical practice has increased the likelihood that a genetically abnormal sperm may be selected for fertilization and participate in the embryo development. In this respect, several negative-impact factors, both genetic and epigenetic in origin, have been identified in embryos following an ICSI procedure (27–30), and there is considerable concern regarding the increased risk of chromosomal abnormalities in infants conceived through ICSI (31–33). Moreover, when considering that the European average "take-home baby" rates are essentially unchanged from a decade ago (34,35), and that, at present, the resulting pregnancy rates are only between 30% and 45% (36–38), it is reasonable to speculate that ICSI might have reached the highest success rate possible as against its technical limitations.

In 1999, seven sperm subcellular organelles (acrosome, postacrosomal lamina, nucleus, neck, axoneme, mitochondrial sheath, and outer dense fibers) were identified and "ultra-morphologically" analyzed by electron microscopy (namely scanning electron microscopy and transmission electron microscopy) (39). As a result, their highly predictive value for male fertility potential was demonstrated. However, analysis of the *sperm organellar morphological characteristics* involved high costs since it had to be carried out only on fixed and stained sperm cells from selected cases of unexplained infertility and repeated assisted reproductive technique (ART) failures. For that reason, a few years later *sperm functional morphology criteria* based on real-time observation of *individual* motile sperm cells under high magnification were developed. The new evaluation procedure, called motile sperm organellar morphology examination (MSOME), used an interference phase-contrast inverted microscope with Nomarski optics that combined objective magnification (100×), a magnification selector (1. 5×), and a video monitor system (video camera plus monitor), which gave a final magnification of 6600× (40). On the basis of historical data, which demonstrated how proper sperm selection improves ICSI outcomes (10,14,41), MSOME, being able to detect subtle sperm morphological malformations, which might remain unnoticed during standard microinjection, and allow the identification of spermatozoa with the best morphology, was introduced to improve the ICSI success rates.

Out of the subcellular organelles examined, sperm nucleus turned out to be the most critical variable affecting the outcome of ICSI (40) particularly in the form of large nuclear vacuoles that were proposed to reflect damage in the nuclear DNA content and organization (42,43). Considering that, at present, we are not able to offer our patients a selection technique whereby spermatozoa used for fertilization are preventively tested for DNA integrity, the assumption that vacuolization of the sperm

nucleus may reflect some underlying DNA defects, which could undermine male fertility potential, is a promising perspective.

Consequently, MSOME evaluation coupled with conventional ICSI gave rise to a new micromanipulation technique called intracytoplasmic morphologically selected sperm injection (IMSI), which is currently one of the most debated issues in the ART field.

The debate on the actual need to introduce such a new method into ART practice as an established treatment tool for infertile couples is lively, and it is not mitigated by the increasing data supporting its clinical effectiveness. Most opponents emphasize practical limitations arising from the technical peculiarities of the procedure including high-cost setting up of the necessary equipment, prolonged handling time of male gametes before fertilization, lack of standardized selection criteria, and problems in managing the ART laboratory. And some even go as far as negating any benefit from its application (44).

Despite those concerns, it is a fact that the implementation of this new method has rekindled interest in the role morphology would have to find functionally competent sperm with the highest fertility potentials. Nevertheless, only after extending its applications and adequate standardization will it be possible to clarify what the actual contribution of IMSI in defeating male infertility is.

EQUIPMENT

The apparatus used for sperm evaluation comprises an inverted microscope equipped with 20×, 40×, and 100× oil immersion objectives mounted preferably on a motorized revolver, and a Nomarski differential interference contrast (DIC) system. The images are captured by a three-charge-coupled device (CCD) video camera and visualized on a monitor screen (Fig. 10.1). Once assembled, the equipment is capable of high magnifications (over 6000×) required to achieve a detailed visualization of the sperm subcellular organelles. DIC is used for detecting optical gradients in the specimen and converting them into intensity differences; it produces monochromatic shadow-cast 3D images that provide information on the

Figure 10.1 IMSI apparatus resulting in the corresponding calculated magnification. *Abbreviations*: DIC, differential interference contrast; CCD, charge-coupled device.

optical path length for both high and low spatial frequencies of the sample.

CALCULATION OF MAGNIFICATION

Calculation of the "total reached magnification" may vary depending upon the system components, but usually it is as follows (Fig. 10.1):

Microscope Magnification
(100 × objective magnification × 1.5 × magnification selector)
×
Video Coupler Magnification
(1 × when no other lenses are inserted)
×
Video Magnification [Monitor diagonal measurement (variable)/CCD chip diagonal measurement (variable)]

For example, to achieve a magnification of 6600×, a 14″ monitor (355.6 mm) and an 8 mm CCD (½-inch) are needed.

CHABLON CALCULATION AND DESIGN

The visualization varies with the technical features of the video camera and monitor, and the related magnification level achieved. The relation between the sperm sizes displayed on the monitor and the real ones has to be established. A chablon, that is a transparent celluloid form that can be superimposed on the monitor to determine whether the sperm under consideration fits the real regular shape and size required for selection (Fig. 10.2), can be calculated and designed using the following formula (see also www.microscopyu.com):

Specimen Real Size = Specimen Length Measured on Screen/Total Magnification

DISH PREPARATION

An IMSI dish could have a variety of designs; however, the basic idea should be to make it fit for the congenial way embryologists work in an IVF lab.

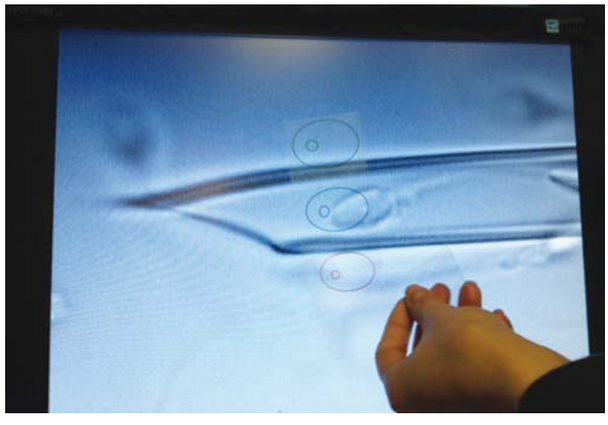

Figure 10.2 The chablon: a transparent celluloid form that can be superimposed on the monitor to verify sperm shape and size.

Due to the application of Nomarski optics, and in accordance with current literature, IMSI requires a sterile glass-bottomed dish featuring the following (Fig. 10.3):

- Observation droplets of sperm culture medium (with HEPES), each containing a different concentration (0–10%) of polyvinylpyrrolidone (PVP) solution and different quantities of sperm suspension according to the quality of the sample. Small bays extruding from the droplet rim are designed to capture the heads of motile spermatozoa. The PVP concentration has to be coordinated with the intensity of the sperm motility in order to slow down the sample and avoid total immobilization.
- Selection droplets of sperm culture medium, where selected, morphologically different sperm cells are positioned after MSOME evaluation. The number of droplets depends on the applied classification that differentiates sperm quality into choices/classes (best and second best (45); Class I–II–II (46)). Hence, every category has a corresponding selection droplet.
- Injection droplets of sperm culture medium that will host the oocytes to be injected in the following ICSI procedure, one for each oocyte available for microinjection. In some cases, embryologists prefer to move the selected sperm to a second plastic dish where the conventional ICSI procedure will take place. All microdroplets are placed under sterile liquid paraffin.

The choice of the glass dish is very important: the less hydrophilic, the more appropriate it is. Thanks to this feature the drop edge tends to remain well defined, which enables better focusing and retard drying of the drops.

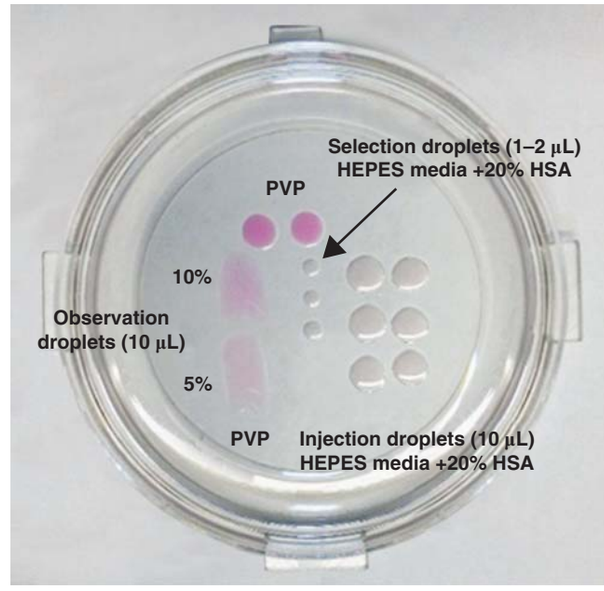

Figure 10.3 An example of a glass-bottomed dish prepared for IMSI (sperm selection and oocyte injection in the same dish). *Abbreviations*: HSA, human serum albumin; HEPES, N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid; PVP, polyvinyl pyrrolidone.

SAMPLE PREPARATION

Freshly ejaculated semen is subjected to standard morphological selection of motile spermatozoa on the basis of a two-layer density gradient system: 1 mL of postejaculated liquefied semen is placed onto the gradient and centrifuged at 375 *g* for 15 minutes at 25°C. The sperm cell pellet is suspended by adding 3 mL of sperm culture medium, and then recentrifuged for 10 minutes. The supernatant is removed and replaced by sperm culture medium to bring the final concentration of motile sperm cells to about 4×10^6 spermatozoa per milliliter. In severe oligozoospermic cases with sperm density below 1×10^6 spermatozoa per ejaculate, liquefied semen are placed onto 1 mL of the low density layer only, and then centrifuged as described above. The final sperm cell pellet is resuspended in 0.1–0.2 mL of sperm culture medium.

MSOME Criteria and Evaluation Procedure

Based on data collected by scanning and transmission electron microscopy (39), the MSOME criteria for normally shaped nuclei are size (average length and width to be 4.75 ± 0.28 µm and 3.28 ± 0.20 µm, respectively), smoothness, symmetry, oval configuration (without extrusion or invagination of the nuclear mass also defined as a regional disorder), and homogeneity of the nuclear chromatin mass containing no more than one vacuole, which occupies less than 4% of the nuclear area (0.78 ± 0.18 µm) (Fig. 10.4). Spermatozoa with abnormal head size are excluded by using the transparent form called chablon (Fig. 10.2). Spermatozoa showing severe malformations, such as a pin, amorphous, tapered, round, or multinucleated head, which can be identified clearly even at low magnifications (200 to 400×), are not assessed by MSOME. Spermatozoa with an uncertain determination are excluded from selection. In order to perform an accurate sperm evaluation, embryologists follow each apparently suitable motile single sperm cell by moving the microscopic stage in the x, y, and z directions until the smallest details are also visualized. Some morphological defects, such as large vacuoles, are revealed only when sperm move. Therefore, motility can be beneficial to morphological observation. On the other hand, static sperm images only allow evaluation of the visible part, leaving some morphological alterations unveiled. Additionally, collaboration between two embryologists analyzing together the same sample at the same time is recommended in order to minimize the subjective nature of sperm evaluation. The amount of time needed to identify the best-looking spermatozoa in a particular sample depends on the quality.

Only motile spermatozoa with morphologically suitable nuclei are retrieved from the observation droplets and aspirated into a sterilized glass (angulated/nonangulated) pipette with a 9 µm inner diameter tip. Sperm cells are then placed into the corresponding selection droplets and finally injected into the oocytes for the traditional ICSI procedure (1) that is performed with a motorized micromanipulator system at room temperature, since prolonged manipulation at 37°C has demonstrated to be detrimental for preserving good sperm quality (47).

CLASSIFICATIONS

For those beginners, IMSI practice differs significantly from theory, being one of the issues that have often limited its wider introduction. The procedure might be perceived as a technically tricky method to learn, extremely complex as against the amount of morphological data to assess and classify. Thus, emerged the need to focus on specific aspects of the sperm morphology and to synthesize that information into classifications drawn from recent advances in the field of infertility and everyday use.

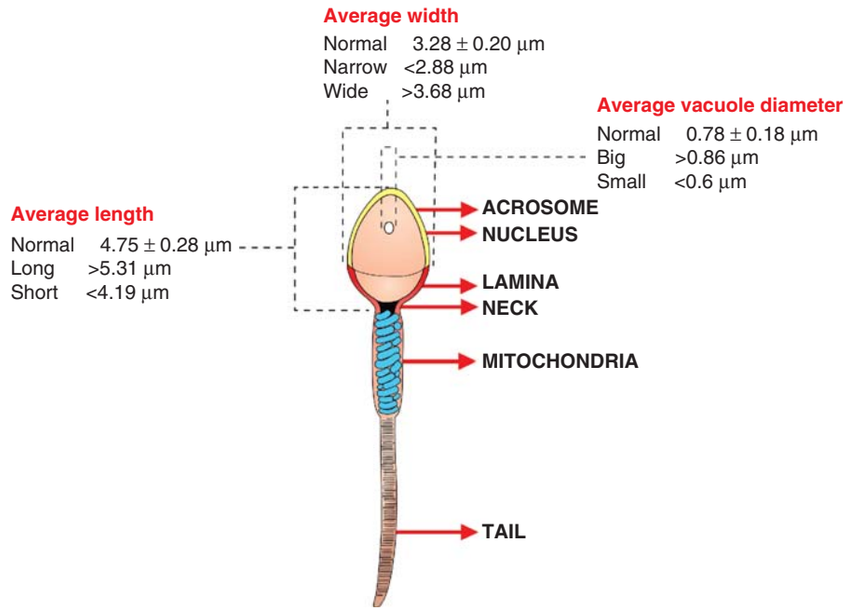

Figure 10.4 Six sperm subcellular organelles evaluated by MSOME criteria (40) and sperm standard dimensions.

Bartoov

Spermatozoa selected by MSOME criteria were evaluated as "best" and "second-best" based on the presence/absence of normal nuclei, respectively, in the test sample. In the second-best group, the following fixed levels of hierarchy were established to facilitate identification of the minimally impaired forms: large/small oval forms, narrow/wide forms, regional disorders, large vacuoles plus normal shape/size, and abnormal forms plus large vacuoles.

Fertilization, top embryo, implantation, pregnancy, and delivery rates per cycle were significantly higher within the "best" group, whereas the abortion rate was significantly lower among the "second-best" choices ($F = 10.5$, $P \leq 0.01$; $F = 4.6$, $P \leq 0.03$; $F = 23.4$, $P \leq 0.01$; $\chi^2 = 15.5$, $P \leq 0.05$; $\chi^2 = 19.6$, $P \leq 0.01$; and $\chi^2 = 5.5$, $P \leq 0.02$, respectively) (45).

Vanderzwalmen

Based on literature demonstrating the existence of a negative association between sperm nuclear vacuoles, natural fertility potential, (48,49) and pregnancy occurrence (43) Vanderzwalmen et al. (50) assume that, for specific morphological malformations, the presence of large vacuoles in the sperm nuclei indicates a more serious damage to the nuclear DNA content and organization than shape or size impairment. Hence, they classify spermatozoa into the following four groups based on the presence, size, and number of vacuoles (Figs. 10.5, 10.6): Grade I: normal shape and lack of vacuoles; Grade II: maximum two small vacuoles; Grade III: more than two small vacuoles or at least one large vacuole; Grade IV: large vacuoles and abnormal head shapes or other defects involving the sperm head base. In a group of 25 IMSI patients, the injection of a total of 442 oocytes with grade I (7%), II (60%), III (20%), and IV (13%) spermatozoa selected at 6600× did not determine any statistically significant difference among the four groups in the number of zygotes and embryo development to day 3. On the other hand, upon joint analysis and multiple comparisons (except for GI vs. GII and GIII vs. GIV), they differed significantly in their development into blastocysts and good-quality blastocysts. Being statistically acceptable to combine groups I and II ($n = 86$), and groups III and IV ($n = 78$), it was observed that, injection with grade I and grade II spermatozoa resulted in a higher rate of blastocyst and good quality blastocyst development compared with compromised spermatozoa (grade III and grade IV) (60.5% vs. 3.8%; $P < 0.001$ and 37.2% vs. 1.3%; $P < 0.001$). Hence, the size and number of nuclear vacuoles demonstrated a substantial negative effect on the embryo development into the blastocyst stage.

Cassuto and Barak

A scoring system was implemented to provide a friendly tool that will allow embryologists to rapidly classify

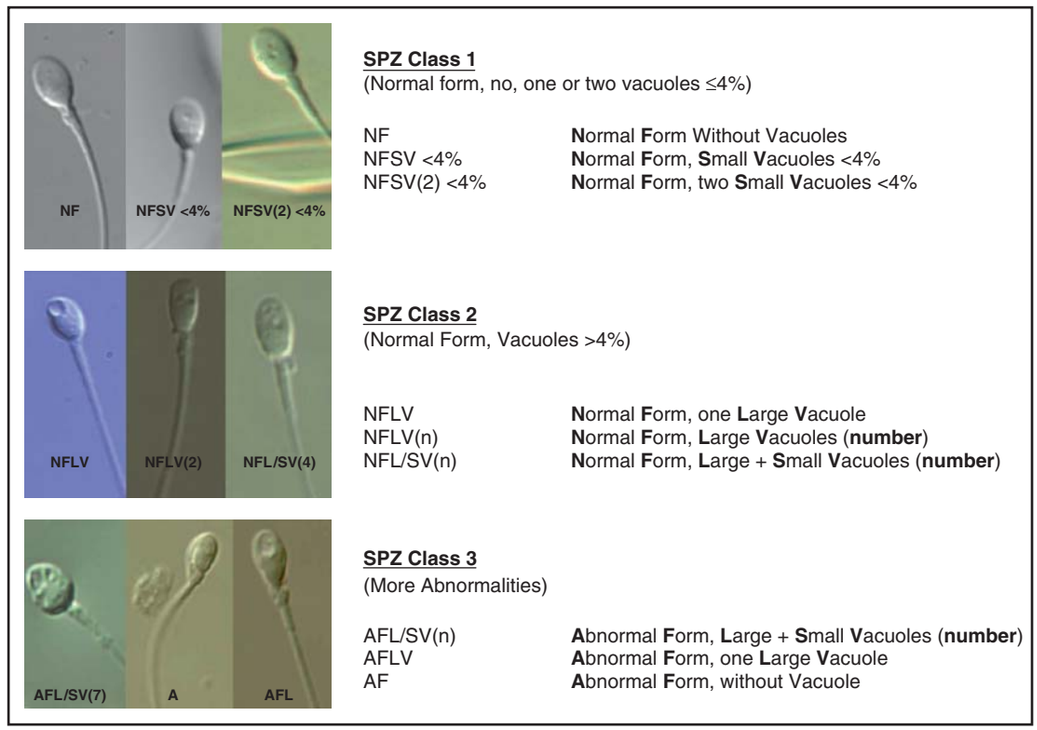

Figure 10.5 Morphological sperm classification at high magnification based on presence, size, and number of vacuoles (50). *Abbreviations*: SPZ, spermatozoa. *Source*: Courtesy of Pierre Vanderzwalmen.

sperm cells just prior ICSI, thus, shortening the prolonged time of the procedure (46). Based on the calculation of coefficients with area under the best ROC curve of 0.618, head shape, presence of vacuoles, and head base shape are identified as the major characteristics for sperm classification. As a result, a fixed total score of 6 points is assigned to sperm with morphologically normal head, vacuoles and base, distributed as follows: Head × 2 + Vacuole × 3 + Base 1. Conversely, 0 points are awarded to each morphologically abnormal sperm structure. Thus, the calculated total range of sperm scoring varies between 0 and 6. Based on the final score obtained, spermatozoa are categorized as follows: Class I (4–6 points) high-quality spermatozoa; Class II (1–3 points) medium-quality spermatozoa; Class III (0 points) poor-quality spermatozoa (Fig. 10.7). When the classification was applied to spermatozoa prior ICSI, following injection the three sperm classes exhibited a statistically significant difference in the fertilization potential: 79.2% for Class I, 63.2% for Class II, and 42.1% for Class III ($P < 0.04$; $\chi^2 = 6.25$). A trend toward a higher rate of development into blastocysts as a function of sperm classification was seen even if the small numbers prevented from reaching a statistical significance.

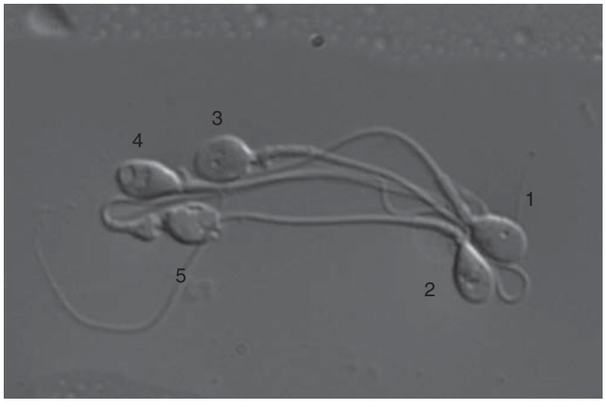

Figure 10.6 Examples of different sizes and numbers of vacuoles according to Ref. 50: 1. Normal shape, one small vacuole; 2. abnormal shape, one small and one large vacuole; 3. abnormal shape, one small vacuole; 4. abnormal shape, one small and one large vacuole; 5. abnormal shape, one small vacuole. *Source*: Courtesy of Pierre Vanderzwalmen.

IMSI REPRODUCTIVE OUTCOMES

Based on the assumption that morphologically severely impaired spermatozoa show reduced fertilization, pregnancy, and implantation rates (9,10), the impact of subtle morphological anomalies detected under high magnification (6600×) on ICSI outcome was investigated to identify the most relevant (40). A total of 10,000 spermatozoa (100 sperm samples, each including 100 spermatozoa) were evaluated. It was observed that in standard IVF-ICSI cycles patients who exhibited less than 20% spermatozoa with normal nucleus—according to MSOME criteria—did not achieve pregnancy. With respect to the ICSI fertilization rate, morphological normalcy of the entire sperm cell—according to MSOME criteria–showed a positive and significant correlation ($r = 0.52$, $P \leq 0.01$) and a very high predictive value (area under the ROC curve, 88%), whereas no association with pregnancy outcome was found. Normalcy of the sperm nucleus (shape + chromatin content), defined by MSOME, was significantly and positively correlated with both fertilization and pregnancy rates ($r = 0.42$, $P \leq 0.01$ and $r = 0.38$, $P \leq 0.01$, respectively). Else, the predictive value of normalcy of the sperm nucleus turned out to be significantly higher (areas under the ROC curve 72% and 74%, respectively). Hence, the authors could conclude that the sperm nucleus is the most important sperm parameter affecting ICSI outcome.

Later on MSOME criteria were applied to choose the morphologically best sperm cell to inject into the oocyte (51): Fifty IMSI couples were compared with 50 ICSI couples within a matched-control study (couples with a similar number of previous ICSI failures). Implantation and pregnancy rates following IMSI were significantly higher, whereas the abortion rate was significantly lower as against the present ICSI trial. In addition, the IMSI attempt produced a significantly higher top embryo percentage as against the present ICSI treatment. Moreover, 12 unmatched IMSI cases with an average of 9.1 ± 1.2 previous ICSI failures achieved a 50% pregnancy rate following their first IMSI trial. Overall, the results demonstrated a significant improvement in the IMSI success rate in couples with at least two previous ICSI failures. According to recent reports, these couples seem to have the worst reproductive prognosis with a dramatic reduction in pregnancy and implantation rates as compared with couples who underwent 0–1 previous IVF failed

Figure 10.7 Cassuto and Barak score system. *Source*: Adapted from Ref. 46.

attempts (52,53). Antinori (54) designed a prospective, randomized, controlled protocol to assess the potential advantages offered by IMSI in the treatment of patients with severe OAT, regardless of previous ICSI failed attempts. The study participants were subsequently split into subgroups by the number of previous failed attempts (subgroup A: no previous attempts; sub-group B: 1 previous failed attempt; sub-group C: ≥2 previous failed attempts). The two different techniques were compared in terms of pregnancy, abortion, and implantation rates.

Pregnancy and implantation rates were statistically higher in the IMSI cycles than in the ICSI group (pregnancy rate: 39.2% vs. 26.5%; $P = 0.004$) (implantation rate: 17.3% vs. 11.3%; $P = 0.007$).

However, cases with two or more failed attempts benefited most from IMSI, with a significant doubling of pregnancy rates (12.9% vs. 29.8%; $P = 0.017$) and a remarkable 50% reduction in the abortion rate (17% vs. 35%). No statistical difference was observed in terms of abortions, but the clinical trend was clearly in favor of the IMSI method in cases with two or more previous failed attempts.

Based on the above results it can be speculated that, in those couples, the male factor could be featured by semen impairment, undetected by conventional diagnostic tools, thus reducing the effectiveness of previous ICSI treatments.

Those conclusions seem to be challenged by a prospective study on 200 couples with a history of at least two previous implantation failures due to mixed infertility factors enrolled by female age matching (≤39 years) in order to compare laboratory and clinical outcomes of IMSI ($n = 100$) and ICSI ($n = 100$) cycles (55). In spite of IMSI showing a positive clinical trend in terms of pregnancy (IMSI: 26% vs. ICSI: 19%), implantation (IMSI: 13.6% vs. ICSI: 9.8%), miscarriage (IMSI: 15.3% vs. ICSI: 31.7%), and live birth (IMSI: 21% vs. ICSI: 12%) rates, it did not produce a statistically significant improvement compared with ICSI. Similar results were obtained also by splitting the study population in subgroups, which could not prove statistically, male factor as a cause of infertility that could benefit the most from IMSI following repeated implantation failures after conventional ICSI.

The same issue was recently investigated by Balaban (56) who tested IMSI application within a prospective randomized study on an unselected infertile population (female age <35 years) to compare the clinical outcome of 87 IMSI and 81 ICSI cycles. Both groups were comparable in terms of fertilization rates and quantity and quality of embryos transferred. Implantation, pregnancy, and live birth rates showed a clinical trend in favor of the IMSI group over the ICSI group (28.9% vs. 19.5% and 54.0% vs. 44.4%, 43.7% vs. 38.3%, respectively; not significant), whereas the IMSI multiple pregnancy rate was significantly higher (34.0% vs. 16.7%; $P < 0.001$). Interestingly, when subgroup analyses on etiology of infertility were performed, male factor couples were seen to benefit more from the IMSI procedure, above all patients with sperm count under 1 million/mL in the basal ejaculate, as shown by significantly higher implantation rates (29.6% vs. 15.2%, $P = 0.01$).

A similar study population was investigated by Wilding (57) within a prospective randomized trial, which involved a heterogeneous population of 232 couples (1–3 years of infertility, sperm count 1×10^6/mL and 20×10^6/mL; female factor included, previous failures not mentioned) to be treated either by IMSI ($n = 122$) or ICSI ($n = 110$). Unlike Balaban's trial, this investigation was able to demonstrate the superior effectiveness of IMSI with respect to the number of good-quality embryos and pregnancy and implantation rates even when applied on an unselected infertile population.

The positive impact on embryo quality (57) and blastocyst development (50) previously reported in the literature, inspired Knez (58) to investigate the clinical role of IMSI on 57 randomized couples (37 IMSI vs. 20 ICSI) with poor semen quality and history of total cleavage arrest after day 5 culture in former ICSI attempts. Probably the lack of a preliminary power calculation, and the relating small size of the sample prevented the clinical outcomes from being significantly improved by IMSI with the exception of the cancellation rate/embryo transfer, which evidenced a significant reduction (IMSI 0% vs. ICSI 27%; $P \leq 0.05$).

The main possible reason for IMSI effectiveness might be the creation of genetically normal embryos with higher chances of implantation and birth, and a decreased abortion risk. To test this theory, Figueira (59) carried out an interesting randomized study on a total of 120 couples (60 IMSI, 60 ICSI) receiving preimplantation genetic screening [fluorescence in situ hybridization (FISH) analysis: x, y, 13, 16, 18, 21, 22] for advanced maternal age (severe OAT excluded). That was the first trial on the chromosomal status of IMSI-derived embryos, and showed the following statistically significant results: lower sex chromosome aneuploidies (IMSI 15% vs. ICSI 23.5%; $P = 0.0139$), lower chaotic chromosomal status with two or more chromosomal numerical abnormalities (IMSI 18.8% vs. ICSI 27.5%; $P = 0.0193$), higher incidence of XX euploid karyotype (IMSI 30% vs. ICSI 21.6%; $P = 0.0326$), and lower risk of transfer cancellation (IMSI 2.5% vs. ICSI 11.8%; $P = 0.0016$). Those outcomes confirm previous results regarding the role of IMSI as an indirect method for rescuing oocytes in patients aged 30 years and older, with impaired capability of repairing the DNA of the injected spermatozoon (46) and might provide a convincing explanation for IMSI effectiveness. However, even if in a recent methanolysis (60), involving a limited number of studies with variable designs, IMSI resulted more beneficial than ICSI in terms of top-quality embryo development, implantation, pregnancy rates and reduction of abortion, the number of RCTs is still too restricted to come to definitive conclusions (Table 10.1).

One of the conceivable expectations from the application of IMSI will be the reduction of congenital anomalies and genetic disorders due to the use of spermatozoa with a normal nucleus, which reduces the risk for DNA defects. That assumption was confirmed by Cassuto (62).

Table 10.1 IMSI Laboratory and Clinical Outcomes

	Study design	Population	Infertility factor/ inclusion criteria	Female age (IMSI group)	Male age	Investigated variables	Main IMSI outcomes (Statistically significant)
Bartoov 2003 (53)	Comparative prospective study matched by previous ICSI failures	50 ICSI vs. 50 IMSI	Male/at least 2 ICSI failures	29.6 ± 3.5	31.7 ± 4.7	FR Embryo quality PR IR AR	Higher PR Higher Embryo quality Higher IR Lower AR
Berkovitz 2006 (43)	Comparative prospective study matched by previous ICSI failures	80 ICSI vs. 80 IMSI	Male/at least 2 ICSI failures	32.3 ± 4.8	NP	FR Embryo quality PR IR AR	Higher PR Higher embryo quality Higher IR Lower AR
Hazout 2006 (61)	Clinical observational	125 previous ICSI failures vs. 125 subsequent IMSI	Mixed/at least 2 ICSI failures	<38	NP	FR Embryo quality PR IR	Higher PR Higher IR
Antinori 2008 (54)	Randomized	219 ICSI vs. 227 IMSI	Male	31.65 ± 3.23	NP	FR PR IR AR	Higher PR Higher IR
Balaban 2011 (56)	Randomized	82 ICSI vs. 87 IMSI	Mixed	29.76 ± 4.03	33.97 ± 5.52	FR PR IR LBR MPR	Higher MPR Higher IR Only in male factor subgroup
Oliveira 2011 (55)	Comparative prospective study matched by female age	100 ICSI vs. 100 IMSI	Mixed	36.8 ± 3.9	39.8 ± 6.2	FR Embryo quality PR IR AR LBR	None
Wilding 2011 (57)	Randomized	110 ICSI vs. 122 IMSI	Mixed	33.6 ± 4.5	NR	FR Embryo quality PR IR LBR	Higher PR Higher embryo quality Higher IR
Knez 2011 (58)	Randomized	37 ICSI vs. 20 IMSI	Mixed	35.8 ± 4.36	NR	FR BDR PR IR	Lower total embryos arrest
Figueira 2011 (59)	Randomized	60 ICSI vs. 60 IMSI	Mixed/First ICSI attempt with PGS for Advanced maternal age	37.3 ± 3.2	43.6 ± 8.9	FR Aneuploidies: X,Y, 13, 16, 18, 21, 22 Transfer cancellation rate	Lower sex chromosomal aneuploidies Higher XX euploid embryos Lower transfer cancellations

Abbreviations: AR, abortion rate; BDR, blastocyst development rate; FR, fertilization rate; ICSI, intracytoplasmic sperm injection; IMSI, intracytoplasmic morphologically selected sperm injection; IR, implantation rate; LBR, live birth rate, MPR, multiple pregnancy rate; NP, not present; NR, not rated; PGS, preimplantation genetic screening; PR, pregnancy rate.

He studied prospectively the follow-up of 1028 children conceived with IMSI (n = 450) and ICSI (n = 578), and compared their outcomes in terms of percentage of major malformations and genetic disorders. The results exhibited that IMSI is associated with significantly fewer birth defects than ICSI (1.77% vs. 4.15%, respectively). Additional evidence comes from the low prevalence (1.5%) of chromosomal abnormalities and birth defects observed by our group on a population of 443 children born after IMSI.

IMSI and DNA Integrity

When Kruger strict criteria were originally introduced (63), it was still not possible to associate poor sperm morphology with fragmented DNA (64,65), whereas following reports found that abnormal spermatozoa negatively correlate with DNA integrity (66,67), especially in case of head malformations (68–70). The potential relationship between sperm morphology and genetic integrity has become very relevant following the introduction of ICSI that gives a chance of fertilization even to those male gametes affected by severe malformations (13,67,71) but it is limited by its low magnification and low resolution of the sperm morphology assessment, which might miss some subtle defects. In both cases, there is considerable concern about the fact that morphologically abnormal spermatozoa with significantly elevated levels of numerical sperm chromosomal aberrations, reactive oxygen species (ROS) production, and DNA chain fragmentation (72,73) would contribute to impair fertilization, embryogenesis, or fetal development. The same considerable concern is still expressed on their contribution to the birth of infants with higher prevalence of chromosomal abnormalities and birth defects over natural conception (74).

Following the introduction of a new visual method applied on conventional ICSI technique called IMSI, sperm characteristics that were undetected by conventional microscopy can be clearly identified. This means that their association with the ICSI outcome can be investigated and can provide reliable evidence that a morphologically normal sperm nucleus is the most important sperm variable showing a close relation with both fertilization and pregnancy rates (40). Based on preliminary, unpublished data by Bartoov's group, which reported a significant negative correlation between the size of the nuclear vacuoles and chromatin stability assessed by sperm chromatin structure assay, and Lee (75), who demonstrated that no increase in chromosome aberrations was found in spermatozoa with large or small heads, it has been proposed that the existence of large sperm vacuoles in the sperm nuclei indicates more damage to nuclear DNA content and organization than nuclear shape or size impairment (43). Furthermore, large nuclear vacuole inoculation resulted in normal, early embryonic development (normal fertilization, development of top-quality embryos, and implantation) followed by impaired embryo survival (low pregnancy and high abortion rates) (43).

In order to prove that large nuclear vacuoles in the sperm cell may reflect some underlying chromosomal or DNA defect, the selection of sperm cells with and without large vacuoles from the same ejaculate and subsequent evaluation by different biochemical methods from an independent analytic system was recommended (43). Therefore, a report by Hazout (61) compared the outcomes of 125 couples with at least two previous ICSI failures and an undetected female infertility factor that received conventional and high-magnification ICSI in two sequential attempts. Following sperm injection into the oocytes without nuclear alterations, a double PR and a 50% decrease in the abortion rate were recorded as against similar cases treated by conventional ICSI. In 72 of 125 study patients, the degree of sperm DNA fragmentation was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method, and the outcomes of high-magnification ICSI were compared in cases with different sperm DNA fragmentation degrees. However, that test was not performed directly with the ICSI-selected sperm samples. A marked rise in clinical implantation and birth rates was observed in patients with normal (<30%), moderately increased (30–40%), and highly (>40%) increased incidence of DNA fragmentation in the ejaculated spermatozoa.

In order to highlight the above assumptions, Franco (76) evaluated the extent of DNA fragmentation (TUNEL assay) and the presence of denatured single-stranded or normal double-stranded DNA (acridine orange fluorescence technique) in spermatozoa with large nuclear vacuoles selected under high magnification compared with those with normal nucleus. The percentage of positive DNA fragmentation was significantly higher ($P < 0.0001$) in large nuclear vacuole spermatozoa (29.1%) than in the normal nucleus group (15.9%). Similarly, the percentage of denatured single-stranded DNA was significantly higher ($P < 0.0001$) in the former (67.9%) than in the latter (33.1%).

So far, a direct relation to DNA quality has not been tested in single selected spermatozoa. With that in mind as a major goal, Garolla (77) evaluated 10 patients affected by severe testicular damage (severe oligozoospermia) by assaying the chromatin structure (sperm DNA integrity by acridine orange; DNA fragmentation by TUNEL assay) and sperm aneuploidies (FISH test) on single immotile sperm cells morphologically selected under high magnification (13,161×). Ten morphologically normal spermatozoa with no vacuoles (group A) and 10 morphologically normal spermatozoa with at least one large head vacuole (group B) were retrieved from each sample. Single cells from group A showed a more physiological status of DNA integrity and DNA fragmentation than cells from group B. Furthermore, no chromosomal alteration was detected by FISH analysis in group-A cells. Moreover, the authors reported that, when morphologically normal spermatozoa with or without large head vacuoles were considered as a single population, the mean results (data not shown) from all tests were significantly better than

those obtained from unselected cells in the first part of the study (all $P < 0.001$).

As a result, a strong correlation between high-magnification morphology, sperm DNA status, and the chromatin origin of nuclear vacuoles visualized by MSOME was inferred, which could be indicative of molecular defects responsible for abnormal chromatin remodeling during sperm maturation, and sperm DNA damage (78), thus compromising ICSI outcomes.

Considering that DIC does may not allow intracellular evaluation (79–81) because of technical limitations, and that detection of chromatin vacuoles in the anterior part of the sperm head requires electron microscopy under a magnification factor of 20,000×, the acrosomal origin of the vacuoles was theorized (82). The first experiment of this study consisted of MSOME evaluation on immotile spermatozoa followed by assessment of the sperm acrosomal status using *Pisum sativum* agglutinin (PSA)-fluorescein isothiocyanate. In most cases (70.9%), the complete acrosome reaction corresponded to regularly shaped spermatozoa with no or minor vacuolization, whereas in sperm with incomplete or missing acrosome reaction the vacuole presence was 60.7%. The second experiment involved evaluation of immotile sperm by MSOME before and after inducing acrosome reaction by ionophore A23587. Vacuole-free spermatozoa increased from 41.2% to 63.8% ($P > 0.005$), so did acrosome-reacted gametes that rose from 17.4 + 7.8 to 36.1 + 12.7 ($P < 0.001$). The last part of the study was performed on motile acrosome-reacting spermatozoa that were analyzed by MSOME. Large protruding blebs, similar to vacuoles when seen upfront, as well as a sort of invagination looking like a vacuole, were seen in the following image. The author came to the conclusion that vacuole-free spermatozoa microinjected during IMSI are mostly acrosome-reacted spermatozoa.

Origin and significance of large nuclear vacuoles were also investigated by other groups. Almeida (83) evaluated 10,000 spermatozoa from 50 men (mean age: 31.4 ± 3.7 years; sperm count: 25.7 × 10^6 cells/mL; motility: 43.7%; Kruger normal forms: 5.5%) undergoing ICSI procedure by some MSOME criteria. A positive correlation was noted between the presence of large and small vacuoles, the level of fragmented DNA assessed by TUNEL test, and the patient's age, whereas no association was seen between chromosomal aneuploidies tested by FISH technique on chromosomes X, Y, 13, 18, 21 and the presence of nuclear vacuoles.

In a recent study (84), transmission electron microscopy was employed to determine the localization of spermatozoa with large vacuoles (defined as those occupying >13% of the head area) from three teratozoospermic, highly vacuolated samples (33%, 18%, and40%). The result showed that their localization corresponded exactly to the nucleus. Then, confocal microscopy coupled with 4',6-diamidino-2-phenylindole (DAPI) immunofluorescence was performed on the same samples and showed that mean DAPI fluorescence intensity has the same evolution in the nucleus and vacuoles, but it is significantly reduced in vacuoles as against the remaining nucleus (83.6 vs. 211.4; $P < 0.0001$).

As a second experiment, in the same trial, fresh samples from 20 teratozoospermic men were used to evaluate acrosome morphology (proacrosin immunolabeling), the level of DNA fragmentation (TUNEL test), and chromatin condensation (aniline blue staining), as well as the appearance of aneuploidies (fish) in both native and vacuolated spermatozoa selected by 6600× magnification. As shown by the results, vacuolated spermatozoa demonstrated a higher percentage of abnormal acrosome morphology (77.6% vs. 70.6%; $P = 0.014$), abnormal chromatin condensation (50.4% vs. 26.5%; $P < 0.0001$), and chromosome abnormalities (7.8% vs. 1.3%; $P < 0.0001$) than the native ones; however, no connection between vacuoles and DNA fragmentation was reported.

A further contribution to investigate the origin and structure of large vacuoles was given by Boitrelle (85). In her study, a population of 15 men with different quality of the sperm samples (five normospermic, five oligo-astheno-teratozoospermic, and five teratozoospermic) was enrolled to provide 30 top and vacuolated (>25%) spermatozoa. Each sperm cell was then compared with unselected sperm cells by using a triple probe procedure: aniline blue staining (condensation), TUNEL assay (DNA fragmentation), and FISH analysis (X,Y,18). The non-condensed chromatin rate resulted significantly higher in vacuolated spermatozoa than in "top" (36.2% + 1.9% vs. 7.6% + 1.3%, respectively; $P < 0.0001$) and unselected spermatozoa (36.2% + 1.9% vs. 25.1% + 3.7%, respectively; $P < 0.01$). DNA fragmentation rates were similarly low for both "top" and "vacuolated" spermatozoa (0.7% + 0.4% vs. 1.3% + 0.4%, respectively; $P = 0.25$). The "top" and "vacuolated" spermatozoa did not differ significantly in terms of aneuploidy rate (1.1% + 0.5% vs. 2.2% + 0.7%, respectively; $P = 0.25$). In the second study phase, atomic force microscopy was used to evaluate 10 "top" and 10 vacuolated sperm cells from two subjects (normozoospermic and oligo-astheno-teratozoospermic, respectively) in order to determine the mean cross-sectional area of the anterior and posterior portion of the head as well as large vacuoles. The vacuoles turned out to be a concavity in the intact structure of the plasma membrane (Fig. 10.8). That finding was further evaluated by 3D deconvolution microscopy analysis on five different samples (two normozoospermic, two oligo-astheno-teratozoospermic, and one with teratozoospermia) having 40 spermatozoa each (equally split between "top" and vacuolated). Labeling of DNA and acrosome was performed by DAPI and PSA lectin staining, respectively; both methods were then combined and evaluated by simultaneous DIC/epifluorescence observations. As shown by the results, a vacuole can be interpreted as a DAPI negative nuclear concavity surrounded by plasma and acrosomal membranes and intact acrosome (Fig. 10.9) linked to failure of sperm chromatin condensation (85). The above findings were recently confirmed by Cassuto (86) within a study on 5200 spermatozoa from 26 OAT men (count 33.2 ± 30.0 × 10^6/mL; motility 41.3% ± 13.2%; WHO normal morphology 26.2% ± 7.4%) with IVF failures, and an equal number of unselected spermatozoa. The chromatin decondensation

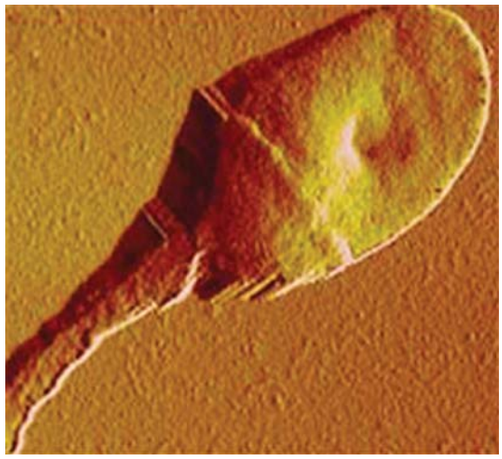

Figure 10.8 A vacuolated spermatozoon observed using Atomic Force Microscopy (AFM) to determine the mean cross-sectional area of both the anterior and posterior portion of the head. *Source*: Adapted from Ref. 85.

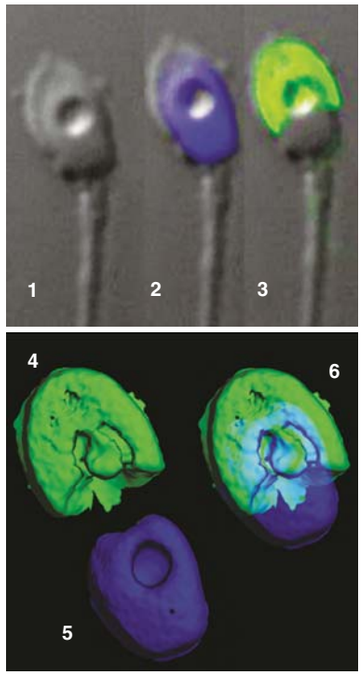

Figure 10.9 Vacuolated spermatozoon, observed under high magnification with Nomarski contrast (DIC): a DIC/DAPI merge (1 and 2) and a DIC/FITC-labeled PSA lectin merge (3). 3D reconstructed images of the same spermatozoa (4–6). DAPI fluoresces blue (4) and PSA lectin fluoresces green (5). Colocalization of fluorescent probes (6). *Abbreviations*: DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast; FITC, fluorescein isothiocyanate; PSA, *Pisum sativum* agglutinin. *Source*: Courtesy of Florence Boitrelle.

rate scored 0 according to his classification (highly vacuolated and morphologically abnormal), and turned out to be twice as high as in the control group (19.5% vs. 10.1%; $P < 0.0001$), whereas DNA fragmentation did not significantly differ between the groups (4.2% vs. 3.7%; P = not significant).

A different theory regarding the real meaning of sperm head large vacuoles and their presumed detrimental effect on sperm DNA integrity was made by Watanabe (87). TUNEL assay applied on a heterogeneous population of 12 men (mainly normospermic: 6/12) detected DNA fragmentation in a similar small percentage of both normal nonvacuolated (3.3%) and normal large vacuolated (3.5%) spermatozoa confirming Boitrelle's observations. Moreover when TUNEL fluorescein images are merged with the corresponding DAPI and DIC ones from the same analyzed sperm, it was observed that DAPI negative/fluorescein negative signals (no nuclear content) can correspond to the localization of large nuclear vacuoles and at the same time sperm with normal shaped nuclei without vacuoles can be DAPI positive/Fluorescein positive (fragmented). Thus the author concluded that large vacuoles are craters that do not reflect abnormalities of the sperm head genome but common physiological variations occurring during human spermatogenesis.

On the basis of a positive correlation between DNA fragmentation and sperm nuclear anomalies (7,57,76,83), even other methods capable of selecting mature spermatozoa with no DNA damage as hyaluronic acid (HA) assay (74,88–92) might facilitate the extraction of sperm cells with normal nuclear morphology during MSOME evaluation. Two different methods, namely a medium with HA (SpermSlow™; MediCult) (92) and an HA-binding assay (PICSI®; MidAtlantic Diagnostics) (93), were applied prior to MSOME analysis to prove their effectiveness in the selection of motile spermatozoa with normal morphology. Results were controversial: while Parmegiani (92) found a significantly higher proportion of nuclearly normal spermatozoa in the HA-bounded group than among those collected from PVP (14.5% vs. 11%, respectively), Petersen (93) did not observe any difference in the normal sperm morphology between HA-bound and HA-unbound spermatozoa (2.7% vs. 2.5%, respectively). Given the different characteristics of the samples involved in the studies (normospermic 94; 6/15 oligospermic 93) and different sperm preparation methods (swim-up 94, density gradient 93) used, further investigations are needed in order to determine the actual contribution of that method to MSOME sperm selection.

CONCLUSIONS

Despite the development of various classifications and methods, a rising number of trials have reported IMSI as having significant clinical benefits in terms of fertilization, embryo quality, pregnancy occurrence, and prosecution for delivery (Table 10.1). The underlying reason for such improvements might be linked to high-magnification (>6000×) assessment offering the chance to detect subtle morphological anomalies that would otherwise be unveiled with conventional methods. Among the different malformations identified by MSOME, those affecting nuclear structure, such as large vacuoles, demonstrated to be highly correlated with male, in vivo and in vitro, reproductive impairment (40,43,50,76). There is still

Table 10.2 Sperm DNA Integrity Tests and Positive Correlation with Vacuoles

	N. subjects	Male age	Parameters	Preparation	Comparison	DNA frag.	Aneuploidies	Abnormal chromatin condensation	Structural chromosome aberration
Franco 2008 (76)	30	NP	Infertile	Density gradients	On single sperm: Normal nucleus vs. LV MSOME criteria	Yes	–	–	–
Garolla 2008 (77)	10	28–37	Severe oligozoos. <1 × 106/mL Testicular damage At least 3 ICSI failures	NP	On single immotile sperm: Normal vs. LV Morphology at 13,000×	Yes	Yes (X,Y,18)	–	–
Almeida 2011 (83)	50	31.4 ± 3.7	Teratozoospermia	NP	On same sample	Yes	No (X,Y,13,18,21)	–	–
Perdrix 2011 (84)	20	36.2 ± 1.3	OAT	Washing DNA frag., aneuploidy: Condensation on native samples Density gradients MSOME DNA Frag. Aneuploidy. Condensation on vacuolated sperm	On single sperm: Native sample vs. LV	No	Yes (X,Y,18)	Yes	–
Wilding 2011 (57)	8	NP	NP	Density gradients	On single sperm: Normal vs. Vacuolated MSOME criteria	Yes	–	–	–
Boitrelle 2011 (85)	15	NP	Mixed; 5 OAT 5 Teratozoo-spermia 5 Normozoo-spermia	Density gradients	On same single sperm: Top SPZ vs. LV SPZ Morphology at 10,000×	No	No	Yes	–
Watanabe 2011 (44)	3	NP	Mixed 1 OAT 2 Normozoospermia	Density gradient followed by swim-up	On single immotile sperm: Normal head vs. LV Morphology at 1000×	No	–	–	–
	12	NP	1 OAT, 5 Asthenozoosper-mia 6 Normozoospermia (2 fertlie donors)	Density gradient followed by swim-up	On single sperm: Normal head vs. LV Morphology at 1000×	–	–	–	No
Cassuto 2012 (86)	26	37.1 ± 7.7	OAT IVF failures	Density gradients	On single sperm: Native sample vs. Score 0	No	–	Yes	–
Franco 2012 (94)	66	37.8 ± 6.5	Mixed	Density gradients	On single sperm: Normal nucleus vs. LV MSOME criteria	–	–	Yes	–

Abbreviations: Frag., fragmentation; LV, large vacuoles; NP, not present; OAT, oligo-astheno-terato-zoospermia; SPZ, spermatozoa.

disagreement on the meaning of sperm head vacuoles since their both acrosomal and nuclear origins have been advocated (82,85). In the former, consumption of the acrosomal components into the acrosomal reaction might induce a process of "devacuolization" (82). On the other hand, large vacuoles might have even a nuclear content of unclear origin. Current literature agrees on the presence of higher decondensed DNA levels corresponding to vacuolated regions (84–86,94) (Table 10.2); however, when DNA fragmentation and chromosomal aneuploidies are assessed to find out a potential correlation with highly vacuolated spermatozoa, results are controversial (Table 10.2) (57,76,77,83–87). In this respect, the wide heterogeneity of the experiments—using unmatchable male populations, different sperm preparations, and unfixed morphological criteria—still seems to make it hard to come to sound conclusions. Nonetheless, mention should be made of the embryologist's skill playing a crucial prerequisite role in the performance of an accurate selection under high magnification. In this respect, few ART units in Japan have been reported to have experienced a dramatic decrease in their fertilization rates in the first six months of IMSI application (95,96). No doubt that the use of new tools (DIC system, immersion oil objectives, and glass-bottom dish), the prolonged manipulation and work overload are potentially challenging. On the other hand, the existence of different morphological classifications, and hence the lack of standardization still constitute a substantial limitation to learning, research, and clinical practice purposes. All those factors create skeptical attitudes toward the future of IMSI in the field of ART (44). However, based on current results, IMSI can be recognized as a promising technique that has fostered deeper understanding of the mechanisms interfering with male fertility potential in both natural and assisted reproduction. Like any other innovation, only extensive use and availability of larger amounts of data will generate strong assumptions on feasibility, effectiveness, and safety of the IMSI technique.

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